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Marine microbial community response to inorganic and organic sediment amendments in laboratory mesocosms

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ABSTRACT

Sediment amendments provide promising strategies of enhancing sequestration of heavy metals and degradation of organic contaminants. The impacts of sediment amendments for metal and organic remediation including apatite, organoclay (and apatite and organoclay in geotextile mats), acetate, and chitin on environmental microbial communities in overlying water and sediment profiles are reported here. These experiments were performed concurrent with an ecotoxicity evaluation (data submitted in companion paper) and X-ray absorption spectroscopy of zinc speciation post apatite amendments. X-ray absorption spectra showed that a modest modification of zinc speciation occurred in amended treatments. Significant changes in both bacterial cell densities and populations were observed in response to amendments of apatite+organoclay, chitin, and acetate. The enriched bacteria and breakdown of these amendments were likely attributed to water quality degradation (e.g. ammonia and dissolved oxygen). Molecular fingerprints of bacterial communities by denaturant gradient gel electrophoresis (DGGE) showed that distinct bacterial populations occurred in overlying waters from different amendments: apatite+organoclay led to the dominance of *Gammaproteobacteria*, acetate enriched *Alphaproteobacteria*, and chitin treatment led to a dominance of *Bacteroidetes* and *Alphaproteobacteria*. In amended sediments, *Firmicutes*, *Bacteroidetes*, and *Deltaproteobacteria* (*Desulfovibrio*) were commonly found with chitin and apatite+chitin treatments. Finally, sulfate-reducing bacteria (e.g. *Desulfovibrio*) and metal-reducing bacteria were also recovered with most probable number (MPN) analyses in treatments with acetate, chitin, and apatite+chitin. These geochemically important bacteria were stimulated by amendments and may play critical functional roles in the metal and organic contaminant remediation process for future investigations of contaminated sediments.

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1. Introduction

Many contaminated sites of concern may be contaminated with both metals and organic contaminants. While sediment dredging and disposal remain important components of current contaminated sediment managements, the use of amendments *in situ* has been proven to be a potential approach to enhance removal of metal and/or organic contaminants from soil, groundwater, and sediments (Anderson et al., 2003; Brown et al., 2004; Istok et al., 2004; Melton and Gardner, 2004; Seager and Gardner, 2005; Werth et al., 2005; Cho et al. 2009). Currently, promising sediment amendments include inorganic (e.g. activated carbon, apatite, organoclay, and geotextile mats containing apatite and organoclay) and organic materials, some of which are also nutrients (e.g. short chain fatty acids and chitin). Organoclays and activated carbon reduced the availability of organic contaminants such as phenols, PCBs, etc. (Mortland et al., 1986; Cho et al., 2009) while phosphates (apatite) helped to immobilize several toxic metals (Ma et al., 1995; Melton and Gardner, 2004; Cao et al., 2009; Paller and Knox, 2010). Geotextiles are porous, synthetic fabrics that could enable the accurate placement of a thin layer of highly sorptive media (i.e., activated carbon, apatite, and organoclays) in the form of reactive mats at sediment sites (McDonough et al., 2007).

Because bacteria are known to influence sediment geochemistry, or assist in the decomposition of many organic contaminants, some researchers have attempted to stimulate indigenous

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bacteria or specific indigenous bacterial populations. Acetate and chitin have been applied for the bioreduction of petroleum hydrocarbon (Kleikemper et al., 2002), degradation of trichloroethylene (Werth et al., 2005), and groundwater dechlorination (Vera et al., 2001). Acetate has been used often for the bioremediation of metals (Anderson et al., 2003; Istok et al., 2004; Chang et al., 2005; Lukas and Hollibaugh, 2001; Lear et al., 2007). Chitin has been used in a few instances for the removal of metals in aqueous solutions (Benguella and Benaissa, 2002; Zhou et al., 2005).

Inorganic amendments mainly involve physical and chemical processes including absorption, adsorption, and transformation. In marine sediments, transition metals form relatively stable insoluble complexes with hydrogen sulfide (HS^-) and carbonate, and/or are sequestered with phosphates and form more stable metal–phosphate complexes, and thus decrease the bioavailability of these metals in natural environments (Brown et al., 2004). Efficient metal immobilization using phosphate relies on increasing the solubility of the phosphate. This process is likely facilitated by phosphate solubilizing bacteria (PSB), which solubilizes phosphate from the undissolved apatite fractions to sequester bioavailable metals. PSB have been extensively studied for agricultural purposes in freshwater systems (reviewed by Rodriguez and Fraga (1999)), but very few studies have examined the microbial capacity for phosphate solubilization in marine systems (Ayyakkannu and Chandramohan, 1971). One reason for this is that marine systems (in general) are not phosphate limited. Recently, remarkable percentages of isolated attached and free-living marine bacteria have been shown to be able to solubilize phosphate compounds with glucose as carbon source (Uzair and Ahmed, 2007). Therefore, we hypothesize that amendment of apatite along with organic carbon source will induce growth of PSB, and subsequently enhance phosphate solubilization and metal immobilization in marine environments.

In contrast to inorganic amendments, organic amendments such as acetate and chitin serve food sources for many microorganisms. Acetate has been used to induce indigenous microbes capable of bioreduction (use metals as electron acceptors) and/or biologically mediated immobilization (e.g. uptake, absorption, etc.) of toxic metals (Anderson et al., 2003; Istok et al., 2004; Chang et al., 2005; Lukas and Hollibaugh, 2001; Lear et al., 2007). Diverse groups of microorganisms have shown interactions with metals and lead to decreased metal solubility and mobility (Brierley, 1990; Tebo, 1995). For instance, iron reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) have been noted for their capabilities in metal precipitation and immobilization, primarily due to their metabolic end-products, such as Fe(II) and sulfide (Lovley, 1993; Barnes et al., 1994; Nealson, 1997; Barton and Fauque 2009). So far, FeRB and SRB have been proven responsible for reduction of many metals including uranium, chromium, manganese, iron, selenate, and arsenate (Tebo and Obraztsova, 1998; Arias and Tebo, 2003a; Anderson et al., 2003; Istok et al., 2004; Chang et al., 2005; Lear et al., 2007). In addition, other bacterial groups, such as *Bacillus* sp. and *Streptomyces* sp. have also been applied in the field and significantly reduced cadmium potentially available for plants (Jezequel and Lebeau, 2008). Furthermore, in an acetate and selenate amendment experiment, Lukas and Hollibaugh (2001) showed even broader phylogenetic bacterial groups responded to added acetate and were responsible for selenate reduction. Thus, indigenous bacteria and enriched bacterial groups may play key roles in the mobilization and immobilization of metals.

Despite the potential importance of the usage of sediment amendments in natural marine systems, there have been few concurrent systematic studies of typical sediment amendments used in marine remediation on the combined responses of the marine invertebrate benthic community, microbial population structures along a horizontal gradient, and corresponding metal speciation to the various amendments. Therefore, the aims of this study were to evaluate the following: (1) the risk or harm to the environment and ecotoxicological effects on macro-invertebrates (Rosen et al., companion submission); (2) what groups of bacteria are enriched through the amendments, and how they potentially influence metal solubility/ecotoxicity; (3) how the amendments affect the metal speciation under natural environments. San Diego Bay reference sediments (Table 2) were used to study the impacts of inorganic (geotextile mats, apatite, and organoclay) and organic (acetate, chitin) amendments on cell densities and population structures of bacterial communities in mescosm experiments. Planktonic and sedimentary microorganisms play central roles in water quality and also, bioremediation. Therefore, bacterial communities were monitored in both overlying waters and in sediments. X-ray absorption spectra were used to examine the metal speciation in San Diego Bay reference sediments under amendment treatments. The information obtained from this current study provides necessary information to aid in decisions for the usage of sediment amendments as remediation strategies in contaminated sediments.

Table 1
Sediment amendments and the concentrations used.

Treatment ID	Treatment description	Amendment concentration (% sed. wt.)
Control	Unamended control	–
Mat	Mat only	–
Mat–Ap	Mat+Apatite	10
Mat–Ap–O	Mat+Apatite+Organoclay	5+5
Ap	Apatite only	5
Ac	Acetate only	5
Ch	Chitin only	2.5
Ap–Ch	Apatite+Chitin	2.5+2.5
O	Organoclay only	5
Ap–O	Apatite+Organoclay	2.5+2.5

Table 2
Physical and chemical characteristics of San Diego Bay reference sediments. Data provided here from Sample SB2433 in Katz (2007).

	San Diego Bay (SD) bulk sediment (μg/g)
% Silt (< 62 μm)	33%
TOC (%)	0.47
Cu	45.6
Zn	132
Cr	41.8
As	6.67
Cd	0.26
Pb	24.8
Ni	10.2

2. Materials and methods

2.1. Amendments and amendment concentrations used

Four different materials were investigated, either singly or in combination: phosfil apatite (rock phosphate mined from North Carolina), PM-199 organoclay (a proprietary granular clay compound marketed by Cetco Remediation Technologies, Hoffman Estates, IL, USA), chitin (from crab shells, practical grade, coarse flakes, Sigma Aldrich, Product #C9213, CAS#1398-61-4), and anhydrous sodium acetate (> 99.9% solid, Sigma Aldrich, CAS#127-09-3). Amendments were either mixed directly into sediment (see Section 2.2) or housed inside reactive core geotextile mat (Cetco). In this study, the geotextile mats were placed beneath a 3 cm layer of uncontaminated sandy sediment from San Diego Bay, CA, to simulate subsequent application of a thin layer cap.

Amendment concentrations were selected based on the range used in recent laboratory and field studies for various types of amendments (Ma et al., 1995; Millward et al., 2005; Cho et al., 2009). The reactive mats were similar to those used by McDonough et al. (2007), but were fabricated into circular shapes with a 3 in. diameter to accommodate the ecotoxicity exposure chambers. The mat core was made from a high-loft polypropylene fiber that was needle-punched into a polypropylene woven geotextile. The high loft fibers had an opening size of 0.85 mm (#20 mesh). The top of the mat was made from a non-woven polypropylene geotextile with similar pore size (~80 μm). Once constructed, the fabricated mats contained apatite and/or organoclay that reflected different dry weight sediment concentrations. The amendment concentrations in the geotextile mats were containing apatite only (10%) and the mats containing organoclay plus apatite (5% of each). The loose amendments concentrations were as follows: apatite in sediment (5%), organoclay in sediment (5%), apatite plus organoclay in sediment (2.5% each), acetate in sediments (5%), chitin in sediments (2.5%), and chitin plus apatite in sediments (2.5% each).

2.2. Sediment preparation

Amendments were mixed into sediment collected from a San Diego Bay reference site (Katz, 2007), and physico-chemical characteristics are summarized in Table 2. Sediment was pressed by hand (without dilution or loss of pore water) through a 2 mm sieve to remove indigenous organisms and large particles prior to use.

For the loosely mixed amendments, the appropriate amounts of sediment and amendment were added to 3.8 L (one-gallon) glass jars, and initially mixed with an impeller mixer attached to a drill motor for 30 min. Following the initial mixing period, all jars were placed on a roll jar mill (US Stoneware, East Palestine, OH, USA) for 48 h for further homogenization. Amended sediments were then added to pre-cleaned, acid-washed, 1 L glass mason jars, which served as the ecotoxicity exposure vessels.

The reactive core mats were leached in flowing filtered seawater (20 μm) for 24 h prior to addition to exposure jars. Mats were placed on the bottom of the jars with the non-woven side up. This was followed by the addition of ~3 cm of SD control sediment.

The jars were set up in replicates of five and continually aerated (~100 bubbles/min) with filtered air. The experiments were conducted at 20 °C (±1 °C) with light and dark (18 and 6 h) cycles. Indicators of overlying water quality (pH, dissolved oxygen, ammonia, salinity, and temperature) were monitored daily (see more details in Rosen et al., companion submission).

2.3. Water and sediment quality measurements

Overlying water quality (pH, D.O., salinity, and temperature) was recorded daily in one surrogate chamber associated with each treatment. Ammonia was measured in both the overlying water and pore water at test initiation and test termination using an ammonia salicylate method (Method 10031, Hach Company, Loveland, CO) with a Hach DR/2400 spectrophotometer. Pore water was collected from the test chambers by decanting the overlying water of the surrogate beakers, and subsequently centrifuging a portion of the remaining sediment at approximately 4000 RPM for 20 min. Unionized ammonia was calculated based on the pH, salinity, and temperature of the overlying water and pore water samples (USEPA, 1989).

2.4. Sample collection for microbiology studies

Because overlying water will affect the macro-benthic community, microbial numbers in the overlying water were analyzed. For overlying water samples, approximately 100 mL of overlying water was collected without disturbing the sediment on days 0, 10, and 28 from each jar, and samples were stored in two 50 mL centrifuge tubes at 4 °C. For sediment sampling, three sediment profiles were collected by sterile pipettes with the ends cut off at days 0 and 28. The sedimentary profiles were evaluated because different groups of bacteria with different metabolic capabilities exist in fairly specific profiles (Arias, et al., 2003b; Edlund, et al., 2008). These cores were transferred to an anaerobic chamber. Sediment was then carefully extruded from the pipette into a large weigh boat, sliced with a wooden toothpick at specific intervals (*T*, top horizon, 0–1.0 cm; *M*, middle horizon, 1.0–2.0 cm; *B*, bottom horizon, 2.0–3.0 cm), and then each specific interval was homogenized. These samples were stored in 10 mL vials under anaerobic conditions using nitrogen gas in the headspace to maintain the anaerobic profiles in the sediment core. The rationale for using the cm scale was based on the observed redox gradient; the sediment changed from a very light brown to darker brown to almost black color within the first 3 cm.

2.5. Most probable number (MPN) analysis

MPN analyses of overlying water and sediment horizon samples (see Section 2.4)—top, 0–1 cm; middle, 1–2 cm; bottom, 2–3 cm—were tested in anaerobically prepared test tubes or microtiter plates (Arias, et al., 2003b). From each specific horizon, 0.5 g of sediment was placed into 1 mL of basal Widdel's medium for marine SRB (without an electron donor or acceptor) followed by serial dilutions. To the Widdel's medium for SRB, acetate (20 mM), lactate (20 mM), and N-acetylglucosamine (20 mM) served as carbon sources (electron donors). Sodium sulfate (20 mM) and sodium thiosulfate (20 mM) were used as terminal electron acceptors for SRB and MRB (metal-reducing bacteria), respectively (Perry et al., 1993; Caccavo et al., 1996). The basic medium (per 1 L) contains 5 g NaCl, 0.4 g $MgCl_2$, 0.3 g KCl, 0.2 g KH_2PO4, 0.15 g $CaCl_2 \cdot 2H_2O$, 1 g yeast extract, 10 mL Vitamin solution (100 × stock) (Kieft et al., 1999), and 10 mL Mineral solution (100 × stock) (Bretschger et al., 2007).

2.6. Cell number determination by epifluorescence microscopy

Overlying water samples were stained by SYBR Gold and observed following the protocol described by Chen et al. (2001) for counting microbial cells. Briefly, 0.5 mL water was fixed with 0.5 mL of 4% paraformaldehyde for 24 h and filtered onto a 0.2 μm pore-size Al_2O_3 Anodisc 25 mm membrane filter (Whatman) with an approximately 10 kPa vacuum. The membranes were stained with 2.5 × SYBR Gold solution (final concentration) in the dark. The stained membrane filters were mounted on glass slides and covered with cover slips. The total bacterial cells were observed and counted under blue excitation (485 nm) on a Zeiss Axioplan epifluorescence microscope (Zeiss, Germany) using 100 × Antiflex Neoflua oil objective lens.

2.7. DNA extraction, PCR, and DGGE

The overlying water samples were filtered by 0.2 μm filters (47 mm diameter) and DNA was extracted with lysozyme, Proteinase K, SDS concomitant with phenol–chloroform extraction, and isopropanol precipitation as previously described (Kan et al., 2006). Three horizons of sediment samples (0.3 g each) were extracted by UltraCleanTM Soil DNA Kit (MO BIO Laboratories) following the manufacturer's instructions. DNA concentration was estimated based on 260 nm absorbance using a Spectrophotometer ND-1000 (Nanodrop).

PCR amplification was performed in a 50 μl reaction containing approximately 25 ng of template DNA, 25 μl PCR Mastermix (Qiagen), 0.5 mM (each) primer, and water (double distilled). PCR program was performed with a Mastercycler (Eppendorf). PCR primers used were 341f (GC) and 907r and the PCR program followed the protocol described by Scäfer and Muyzer (2001). Agarose gel electrophoresis was used to detect and estimate the size of PCR amplicons.

In order to reduce sample numbers, PCR amplicons from replicates of water and sediment samples were pooled before loading on DGGE. Prior to pooling, however, a DGGE was run on the individual samples to ensure acceptable reproducibility (results not shown). DGGE was performed as previously described (Kan et al., 2006), except the linear denaturant gradient was 40–70% instead of 40–65%. Briefly, DGGE was performed using a DcodeTM Universal Mutation Detection System (Bio-Rad) and similar amount of PCR products were loaded on a 1.5 mm-thick vertical polyacrylamide gel with a linear gradient of the denaturants urea and formamide. Electrophoresis was performed at 60 °C in 1 × TAE buffer, and a voltage of 75 V was applied for 16 h. The DGGE gel was stained with SYBR Gold and photographed (Øvreås et al., 1997) with a CCD camera mounted on a UV transilluminator (UVP).

2.8. DGGE band sequencing and phylogenetic analysis

Because DGGE is a semi-quantitative approach, only band presence and absence were used for comparisons by Gelcompar II (Applied Maths). Dominant bands were excised from DGGE gels and incubated in diffusion buffer (0.25 M ammonium acetate, 10 mM magnesium chloride, and 0.1% SDS) at 50 °C for 30 min. One μl supernatant was used to reamplify the band. PCR products were purified by ExoSAP-IT (USB) and sequenced with primer 341 f (no GC) using Bigdye-terminator chemistry by ABI PRISM3100 Genetic Analyzer (Applied Biosystems).

All sequences were compared with GenBank database using BLAST, and the closest matched sequences were obtained and included in the downstream phylogenetic analysis. Phylogenetic trees were constructed using MacVector 10.0 software package (MacVector Inc.). Briefly, sequence alignment was performed with the program CLUSTAL W. Evolutionary distances were calculated using Jukes–Cantor method (Jukes and Cantor, 1969) and distance trees were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987). Bootstrap values were obtained based on the analysis of 1000 resampling datasets. Sequences of the partial 16S rRNA genes of representative DGGE bands have been deposited in the GenBank database under accession numbers GU938714–GU938760.

2.9. X-ray absorption spectroscopy (XAS) speciation analysis

Experiments were conducted at the Materials Research Collaborative Access Team's (MRCAT) beamline 10-ID, Sector 10 located at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Argonne, IL. The electron storage ring

operated at 7 GeV in top-up mode. A liquid N_2 cooled double crystal Si(1 1 1) monochromator was used to select incident photon energies and a platinum-coated mirror was used for harmonic rejection. The beam energy was calibrated by assigning the first derivative inflection point of the K_α-absorption edge of a zinc metal (9659 eV) foil. The samples were prepared as thin pellets with a hand operated IR pellet press and the samples were secured by Kapton tape. The zinc references were diluted with boron nitride to 1000 mg kg^{-1} and formed into pellets. Reference materials examined include hopeite ($Zn_3(PO_4)_24H_2O$), smithsonite ($ZnCO_3$), Zn–Al layered double hydroxide with nitrate and silicate interlayers, $Zn(OH)_2$, ZnO, sphalerite (ZnS), zinc sorbed to ferrihydrite, zinc sulfate, aqueous zinc nitrate, franklinite ($ZnFe_2O_4$), willemite (Zn_2SiO_4), hemimorphite ($Zn_4Si_2O_7(OH)_2H_2O$), and gahnite ($ZnAl_2O_4$). Five XAS spectra were collected in fluorescence mode at room temperature from −200 to 1000 eV relative to the absorption edge position of Zn with a Canberra multielement detector. The I_o chamber was filled with N_2 while the I_t detector (for reference materials) contained approximately 60:40 Ar:N_2.

The collected spectra were analyzed using the Athena software program in the computer package IFEFFIT (Ravel and Newville, 2005) for data reduction and data fitting. The five individual spectra for each sample were averaged followed by subtraction of the background through the pre-edge region using the Autobk algorithm and normalized to an atomic absorption of one. The data were converted from energy to photoelectron momentum (k-space) and weighted by k^3. Identification of zinc phases in the sediment samples was accomplished by principal component analysis (PCA) and linear combination fitting (LCF) of the sediment XAS spectra relative to the known reference spectra.

3. Results

3.1. Stimulation of bacterial growth and microbial cell densities in amendments

Bacterial cell counts were performed on Days 0, 10, and 28 to coincide with the ecotoxicology sampling. Cell counts and fluorescence microscopy observations indicated that among all the treatments, with the exception of those containing acetate, chitin, apatite+chitin, and apatite+organoclay, microbial cell numbers did not change significantly over time (Fig. 1A and B). From Fig. 1B, the bacterial cell densities became stable by day 10 in all the amendments, continuing through day 28.

The acetate treatment stimulated the largest bacterial bloom with the final cell density of $\sim 1.34 \times 10^8$ cells/mL, which was 3 orders magnitude higher than the control (2×10^5 cells/mL). Chitin only and apatite+chitin treatments reached the cell density of $\sim 7.56 \times 10^6$ cells/mL and $\sim 5.64 \times 10^6$ cells/mL, respectively (Fig. 1A). The apatite+organoclay treatment also induced bacterial growth, with a final cell density of 1.76×10^6 cells/mL, slightly less than 10 times the control. In contrast, the cell numbers from other amendments remained similar to the controls, with a final cell density of $\sim 2.6 \times 10^5$ cells/mL.

In addition, different treatments stimulated distinct species of microbes based on the morphology as shown in Fig. 1B. In the acetate treatment, most of the microorganisms were rod-shaped single cells, while in chitin and chitin+apatite treatments, multicellular filamental forms like trichomes were present (Fig. 1B). One interesting difference was observed between chitin only and chitin+apatite treatments: linear multicellular filaments consisting of long-rod-shaped cells in chains were observed in chitin only but were not detected in chitin+apatite amended sediment.

3.2. Microbial community in overlying waters

To determine if the sediment amendments had a great effect on an overlying water column (for example, a static water environment such as a holding pond), the bacteria were counted in the overlying water. As expected, the inorganic amendments applied alone in this study had little or no impact on the microbial populations in overlying water. For example, the mat only, dispersed organoclay, and dispersed organoclay+apatite were not significantly different from the control samples. Worthy of note, the inorganic

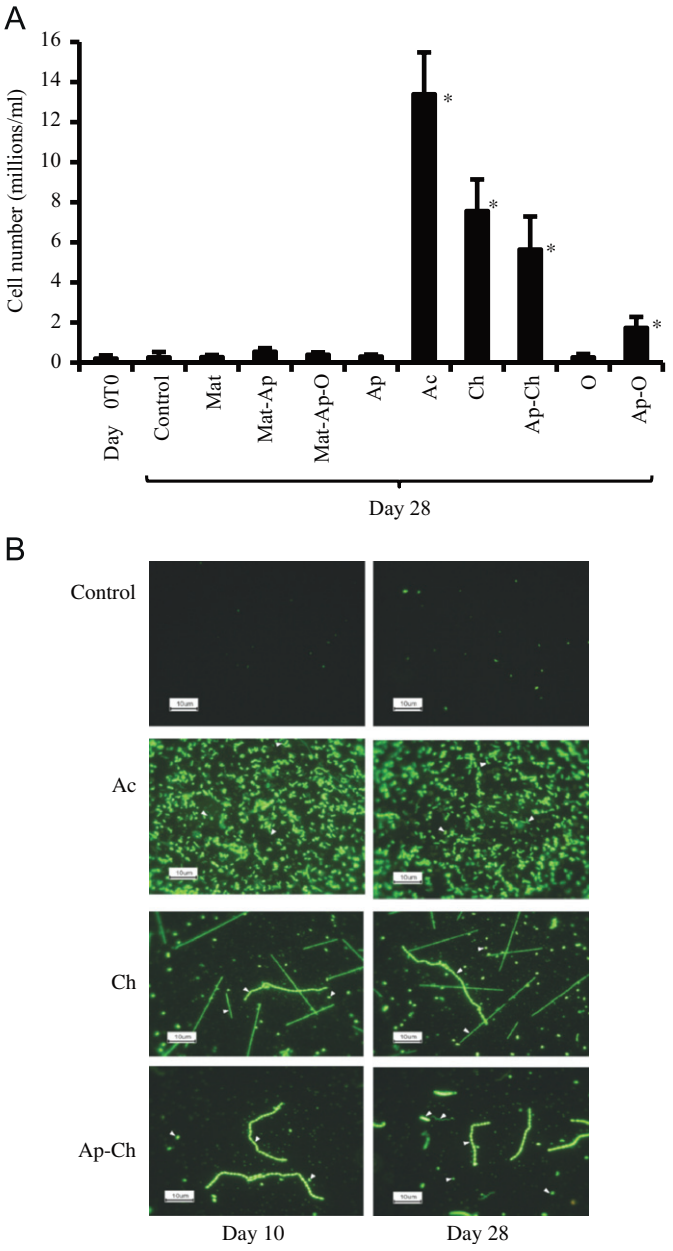

Fig. 1. Enriched microbial cells during the amendment experiments. (A) Bacterial cell counts of overlying water on day 0 (T0) and day 28. Due to the high cell density, counts for treatment with acetate (Ac) were diluted 10 times. (B) Epifluorescence microscopic observation of planktonic bacteria with amendments of acetate, chitin, apatite, and chitin on day 10 and day 28. Other amendment treatments showed similar results as control. Error bars represented the standard deviation ($n=10$). *, significant difference compared to the control (unpaired t-test, $p < 0.05$)

amendments of mat material containing apatite versus dispersed apatite had a few distinct differences. The mat+apatite and apatite only had a common dominant band of *Rugeria* sp. (Fig. 2, Mat–Ap and Ap, bands 2 and 7) observed in the control. However, the mat+apatite contained a *Roseobacter* (Figs. 2, band 3) as a second dominant band relative to the *Sulfitobacter* (Fig. 2, band 4) observed in the dispersed apatite. The mat+apatite+organoclay (Fig. 2, Mat–Ap–O) differed in that the dominant band was *Pseudoalteromonas* spp. (bands 5 and 6). In the mesocosms with different geotextile amendments, distinct bacterial groups were observed in the water column, indicating that the amendments diffused out of the mat material and were capable of causing an effect on the microorganisms in the water column.

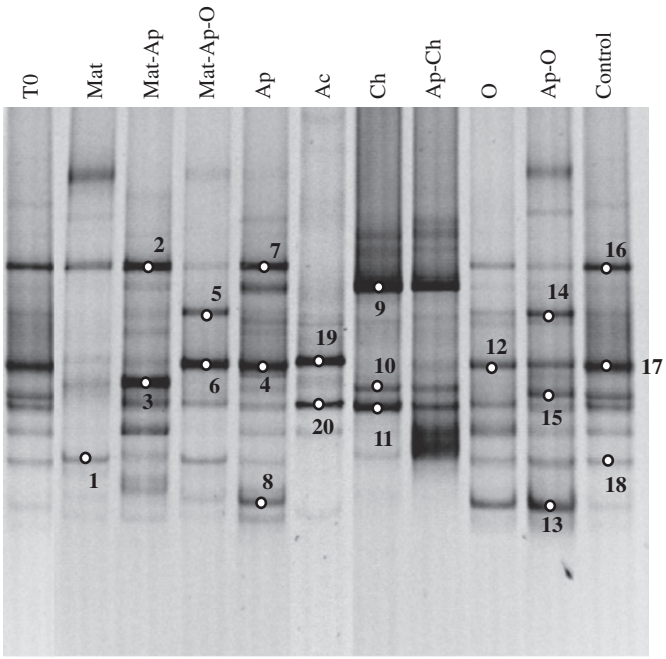

Fig. 2. DGGE fingerprints of bacterial community from overlying waters on day 28. Labels were the same as Table 1 and Fig. 1. Bands 1–20 were selected and excised for sequencing.

In contrast, the acetate and chitin amendments (Ac and Ch in Fig. 2) shifted the bacterial population structures significantly. For example, in the acetate treatment, two bands commonly found in controls, 16 and 17, disappeared while two new bands, 19 and 20, for uncultured Alphaproteobacteria became dominant. Chitin also introduced significant changes with bands 9, 10, and 11 becoming the dominant bands (Fig. 2). These bands represent Bacteroidetes and two unidentified Alphaproteobacteria. The same three bands were seen when apatite and chitin were used together (Ap–Ch). Combined amendments impacted bacterial communities and induced some bacterial groups to appear or disappear in corresponding treatments (Mat–Ap, Mat–Ap–O, and Ap–O in Fig. 2).

Sequencing of the selected DGGE bands and phylogenetic reconstruction confirmed that amendments stimulated distinct bacterial groups from the controls. The control treatments mainly contained bacterial groups of *Sulfitobacter* sp. (band 17) and *Ruegeria* sp. (bands 16 and 18) (Fig. 3). Besides the shared *Sulfitobacter* and *Ruegeria* groups, *Pseudoalteromonas* and *Halomonas* (Gammaproteobacteria) and *Croceibacter* (Bacteroidetes) were obtained in apatite+organoclay and mat+apatite+organoclay amendments (bands 5, 13, 14, and 15, Figs. 2 and 3). In contrast, acetate only amendment induced Alphaproteobacteria (roseobacterial groups) (bands 19 and 20), while chitin and apatite+chitin stimulated *Phaeobacter*, *Roseobacter* (bands 10 and 11), and Bacteroidetes (band 9).

3.3. Bacterial communities in sediments versus overlying water

Due to the significant effects of amendments on microbial population structures within overlying water, we chose acetate only, chitin only, and apatite+chitin treatments to compare the bacterial communities between water and 3 sediment horizon depths. DGGE band patterns indicated that the stimulated bacteria in overlying water and in sediment were similar (Alphaproteobacteria and Bacteroidetes) for acetate amendments (Fig. 4, Ac), but were quite different for chitin and apatite+chitin treatments (Fig. 4, Ch, Ap–Ch). For example, Alphaproteobacteria and Bacteroidetes were dominant in overlying waters from chitin and apatite+chitin. However, Deltaproteobacteria (*Desulfovibrio*, bands 25, 27, and 37), Firmicutes (bands 21, 24, 33, 34, and 36), and Bacteroidetes (bands 29, 30, 40, and 41) were the primary retrieved phylotypes within the 3 sediment horizons analyzed (Figs. 4 and 5).

3.4. MPN analysis

Compared to the control and other treatments, SRB and MRB were significantly increased in all three horizons of sediments amended with acetate, chitin, and apatite+chitin. The MPN analysis confirmed that the three measured sediment horizons (0–1, 1–2, and 2–3 cm) showed a 10–100 fold increase in numbers of MRB and SRB versus the control. SRB and MRB were also recovered in overlying water samples from the treatment with acetate, where the water levels near the sediment/ water interface became anaerobic (Fig. 3 in Rosen et al., companion submission) during the experiments.

3.5. X-ray absorption spectroscopy speciation analysis

Fig. 6A showed the XAS spectra of the reference, relatively uncontaminated sandy sediment samples from San Diego Bay, CA, with unamended, apatite, apatite+chitin, and chitin treatments. XAS was focused on metal analysis; therefore, XAS was not performed for the organoclay or geotextile mats amendments. Also, because acetate would not be used in a field due to its expense, but chitin may be used due to its formation of acetate as a by-product (Bassler et al., 1991), chitin was evaluated as a surrogate of an acetate treatment.

The low concentration of Zn in the sediment material is reflected in the relatively low edge step (absorption intensity) (Table 3). Principal component analysis (PCA) identified five suitable components for linear combination fitting (LCF) validity. These components include hopeite ($Zn_3(PO_4)_2 4H_2O$), smithsonite ($ZnCO_3$), zinc sorbed to ferrihydrite, franklinite ($ZnFe_2O_4$), and gahnite ($ZnAl_2O_4$) (Fig. 6B). The accuracy of LCF results is estimated to be $\pm$10% (Scheckel et al., 2005). However, given the relative concentration of the samples presented here, $\pm$15% accuracy may be a better estimate.

In all cases, the two dominant forms of Zn in the unamended and amended sediments are gahnite and Zn sorbed to an iron oxide (ferrihydrite) (Table 3). With apatite as an amendment, a small portion of the Zn-phosphate mineral, hopeite, was observed. Likewise, the chitin amendment, which demonstrated a positive response to the microbial community, was noted to have an increase in $ZnCO_3$ species.

4. Discussion

This work was performed alongside a companion paper (Rosen et al., this issue) to address the potential effects of sediment amendments used to decrease organic and inorganic pollutants in sediments on the macro-benthic marine communities. In the Rosen paper, effects such as decreased oxygen levels in the system or increased growth of the polychaete worm were likely caused by the microbiology in the mesocosms. This paper demonstrates how the sediment amendments affected the bacteria, which in turn had an effect on the benthic biota, and the metal speciation under natural environments.

4.1. Apatite amendment and phosphate solubilizing bacteria (PSB)

The inorganic amendments did influence the microbial cell densities or community structures when combined with other

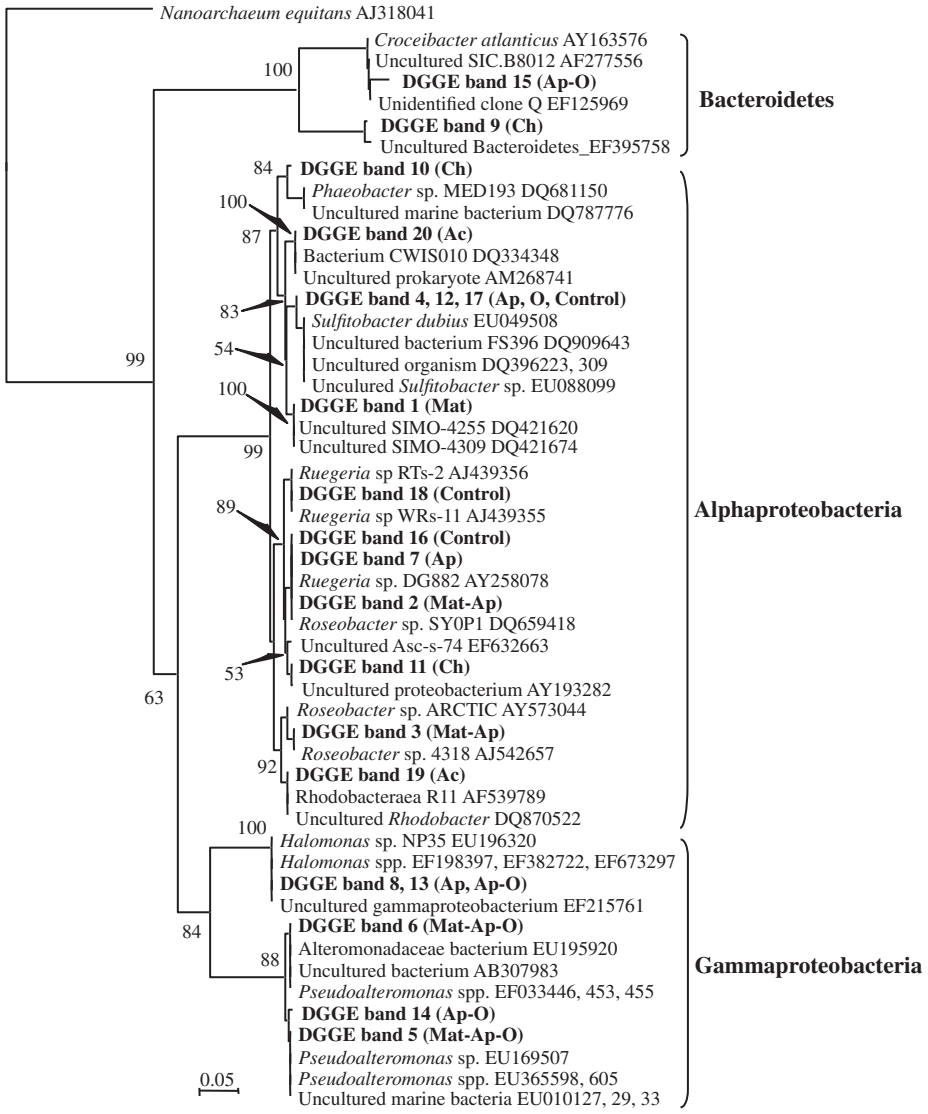

Fig. 3. Phylogenetic analysis of DGGE band sequences obtained from Fig. 3. Most closely related representatives from GenBank were included. Bootstrap values were calculated based on 1000 resampling datasets. For clarity, only bootstrap values relevant to the interpretation of groupings were shown. Scale bar indicated the number of substitutions per site.

amendments. The chitin+apatite and apatite+organoclay significantly increased the microbial cell densities in water columns (Fig. 1A) and chitin+apatite enriched different bacterial groups, including Gammaproteobacteria, Deltaproteobacteria, Firmicutes, and Bacteroidetes in sediments (Figs. 4 and 5). Certain groups of bacteria have been proved to be capable of producing sulfides (e.g. Deltaproteobacteria (Barnes et al., 1994; Barton and Fauque, 2009)) and carbonates (e.g. Gammaproteobacteria and Firmicutes (Rivadeneyra et al., 1994a,b)) that quickly bind bioavailable metals. Under the chitin amendment, the XAS analysis demonstrated an increase in the smithosonite, $ZnCO_3$ species (Table 3 and Fig. 6). Under more oxic conditions, phosphate abiotically solubilized from the fine-grained fraction of the apatite amendment rapidly sequestered bioavailable metals from pore waters to form metal phosphates, a process that was also observed by others (Ma et al., 1995; Laperche et al., 1996). The XAS speciation results for Zn in unamended and apatite amended sediments for this study also showed occurrence of mineral Zn-phosphate (hopeite) (Table 3 and Fig. 6), which were in line with previous observations (Scheckel et al., 2011). Metal phosphates are typically more thermodynamically stable than the metal sulfides (Nriagu, 1974), so over time there should be a conversion of transitory metal sulfides to metal phosphates where these mixed amendment systems helped provide a more efficient and permanent metal sequestration.

Recent evidence suggests bacteria such as *Beggiatoa* sp. and *Thiomargarita* sp. may promote the precipitation of apatite minerals via the enhancement of phosphate gradients (Schulz and Schulz, 2005). Furthermore, additions of organic materials (as carbon sources) have been shown to enhance the bacterial growth rate and phosphate solubilization (De Souza et al., 2000). Bacterial involvement has been invoked to explain the formation of phosphorites (natural apatite mineral deposits) in both present day (Baturin, 1983) and ancient (Reimers et al., 1990; Leather, 1993) environments. In freshwater systems, strains of *Bacillus*, *Rhizobium*, and *Pseudomonas* have been identified as abundant phosphate solubilizers (Rodriguez and Fraga, 1999). More recently diverse bacterial groups (i.e., *Serratia*, *Shewanella*, *Escherichia coli*, *Vibrio*, and *Proteus*) have been shown to be capable of solubilizing phosphate compounds that were previously considered to be insoluble (Uzair and Ahmed, 2007). Some of these groups of bacteria (*Pseudomonas*, *Shewanella*, and

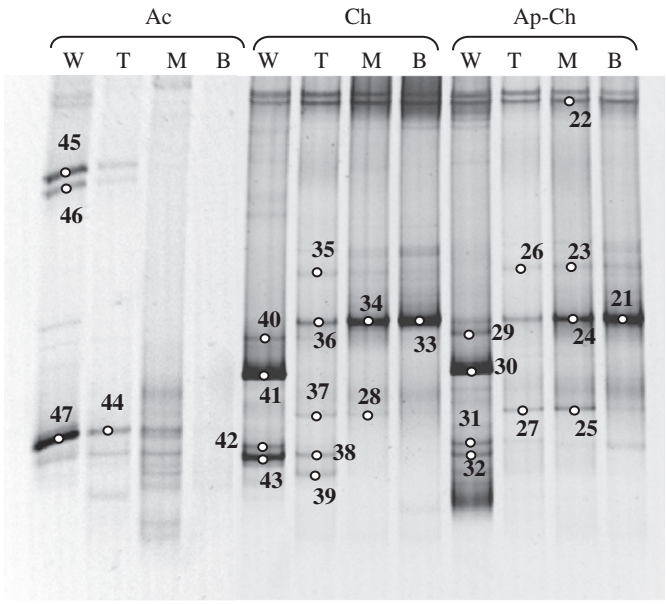

Fig. 4. DGGE fingerprints of overlying waters and sediment bacterial communities collected on day 28. Amendments: Ac, acetate only; Ch, chitin only; Ap–Ch, apatite+chitin. Water samples (W) and three horizons of sediments were included in the analysis. T=top horizon, 0–1.0 cm; M=middle horizon, 1.0–2.0 cm; B=bottom horizon, 2.0–3.0 cm. Bands 21–47 were excised for sequencing.

Vibrio) are also common to ocean systems and therefore we expected they might also be capable of phosphate solubilization in marine systems. In this study, however, the molecular analysis (culture-independent approach) did not retrieve any of the bacteria as discussed above. The enriched bacteria belonged to diverse bacterial groups including Gammaproteobacteria, Deltaproteobacteria, Firmicutes, and Bacteroidetes; therefore, bacteria that can solubilize phosphate may be present in these sediments. This is likely due to the discrepancy between cultivation and molecular analyses, that isolates represent a negligible fraction of natural communities. Future work in characterizing the phosphate solubilization of those bacterial groups recovered from molecular analyses will fill this obvious gap and help fully understand the geochemical processes that will occur.

4.2. Effects of acetate and chitin amendments

Acetate and chitin are two commonly used organic amendments. Acetate is a two-carbon short chain fatty acid and serves a general food source for most of the microbes including bacteria, archaea, and even eukaryotic microbes. The key enzymes (i.e., acetate kinase and phosphotransacetylase) of the acetate metabolic pathway are widely distributed in bacteria and thus acetate serves as the best carbon source to stimulate the growth of indigenous bacteria in natural environments (Ingram-Smith et al., 2006). In contrast, chitin is an abundant structural polysaccharide produced by many marine organisms and it is a (1→4)-β-linked homopolymer of N-acetylglucosamine (NAG). The primary breakdown products of chitin are acetate and fructose, both of which are excellent carbon sources for anaerobic and facultative microorganisms capable of metal reduction (Bassler et al., 1991). Chitin can be purchased as waste-product from the crab and shrimp industry; therefore, it serves as an excellent cost-effective source to stimulate bacteria that grow in response to acetate.

Acetate and chitin induced significant increase in bacterial cell numbers and shifted the bacterial community composition in both water and sediments. Amendments of acetate, chitin, or chitin+apatite induced more diverse groups of *Roseobacter* in overlying waters, which were not dominant in the control (e.g. bands 3, 10, 11, 19, and 20 in Figs. 2 and 3). In marine environments, *Roseobacter* was a major phylogenetic group (about 5–30% of total) and they were widely distributed across a wide gradient of environments (reviewed in Buchan et al. (2005)). Members of *Roseobacter* have been found to be free-living, particle-associated, or in a symbiotic relationship with other living organisms (Buchan et al., 2005). One interesting physiological feature of *Roseobacter* was transformations in the biogeochemical cycling of sulfur. *Roseobacter* spp. harbored the ability to transform both organic (degradation) and inorganic (oxidation) forms of sulfur, including elemental sulfur, sulfide, sulfite, and thiosulfate (Moran et al., 2003). Many metal ions react with different forms of sulfur (e.g. sulfide) and form relatively stable compounds such as metal sulfides. Although little is known about the direct physiological roles of *Roseobacter* on heavy metals or organic contaminants, comparative genomic studies have shown that *Roseobacter* genomes contain common genes associated with metal toxicity, such as ABC-type transporter genes, copper resistance genes (copA, copB), and arsenate reductase gene (arsC) (Moran et al., 2007). These observations and the fact that organic amendments stimulated the growth of *Roseobacter* suggest that the versatile physiological features of this group of Alphaproteobacteria deserve further study.

Organic amendments also stimulated distinct bacterial communities in sediments in comparison to overlying water. For instance, Firmicutes and Bacteroidetes were dominant in the three horizons of chitin-amended sediments. Firmicutes and Bacteroidetes are two widely distributed bacterial groups, which are common to soils, sediments, seawater, and animal guts. To date, it is not clear if these two bacterial groups are centrally involved in metal bioremediation, a fact that is partially attributed to the difficulty of cultivation of environmental microbes in the laboratory. For instance, our DGGE band sequences primarily matched with uncultured bacterial phylotypes in the GenBank (Fig. 6). However, the high occurrence of these two groups of bacteria under heavy metal environments (Akob et al., 2006; Garau et al., 2007) suggests that (1) these two groups of microorganisms may be well adapted to contaminated sites, and (2) if not directly, these bacteria may cooperate with other microorganisms to facilitate the process of heavy metal immobilization or bioremediation in natural environments.

Based on MPN analyses, it is clear that the abundance of SRB was increased by both acetate and chintin amendments compared to controls. SRB are anaerobic microorganisms that commonly use sulfate as the terminal electron acceptor. Besides high production of sulfide, SRB are also capable of mediating electron flow to metals via dissimilatory reduction of metals. Therefore, SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS^- (Arias and Tebo, 2003a; Muyzer and Stams, 2008; Barton and Fauque, 2009). In our DGGE analysis, a group of SRB (*Desulfovibrio*) was present in chitin treatments but no SRB was recovered in acetate treatments. This observation agrees with a previous study that SRB were detected in petroleum hydrocarbon contaminated aquifers by fluorescence *in situ* hybridization (FISH) and sulfur isotope fractionation, but not via DGGE (Kleikemper et al., 2002). In fact, we cannot completely rule out the occurrence of SRB other than *Desulfovibrio* in amendments because PCR–DGGE only reveals the dominant populations and misses the minor groups that have never reached densities to be detected by PCR–DGGE (Muyzer et al., 1993; Kan et al., 2006). Given the fact that minor groups of microbes could have a major geochemical impact, the results underscore the importance of more detailed cultivation-linked and cultivation-independent molecular techniques such as cloning, pyrosequencing, and metogenomics for future investigations.

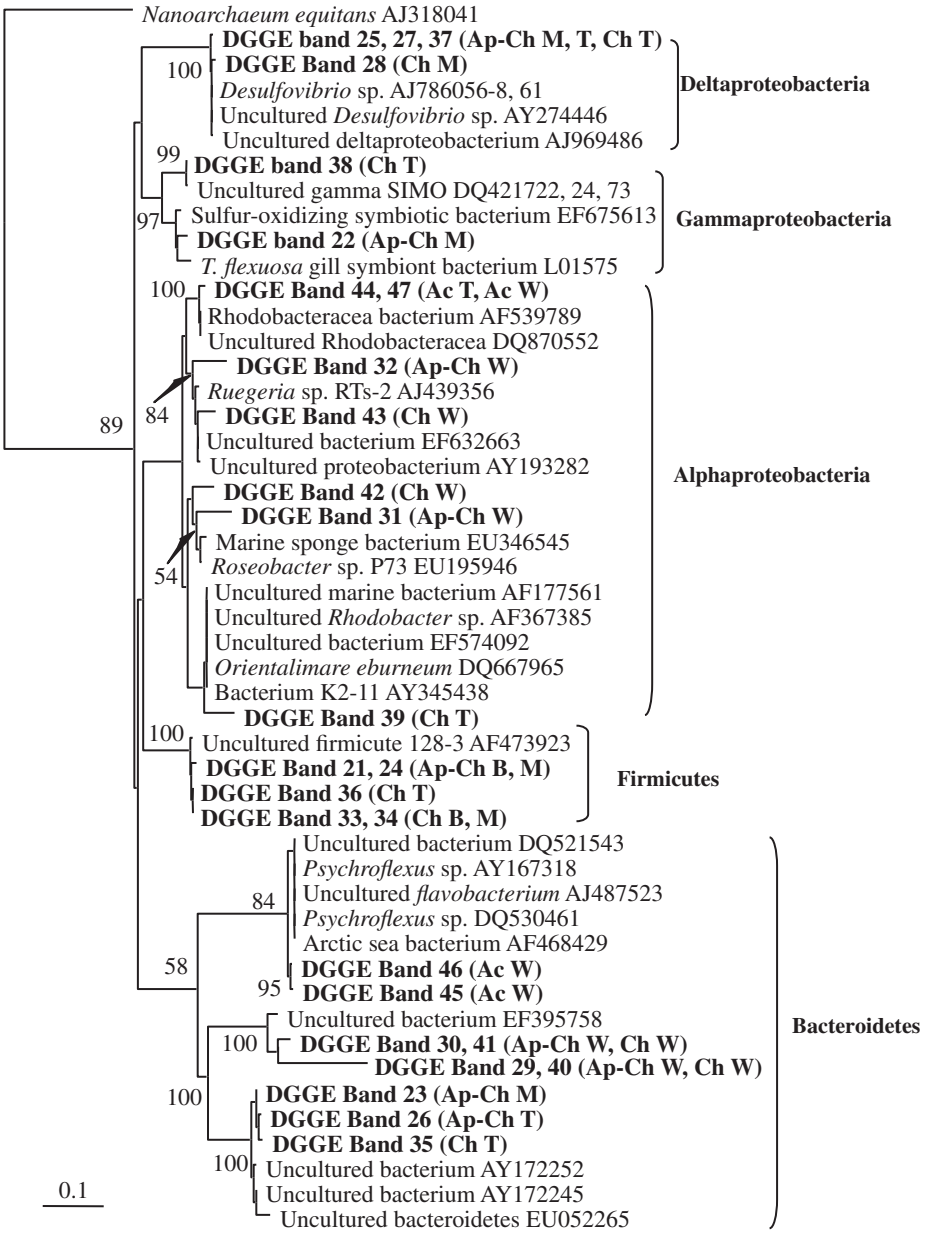

Fig. 5. Phylogenetic analysis of DGGE band sequences obtained from Fig. 4 and mostly closely related representatives from GenBank. Bootstrap values were calculated based on 1000 resampling datasets. For clarity, only bootstrap values relevant to the interpretation of groupings were shown. Scale bar indicated the number of substitutions per site.

4.3. Water quality and ecotoxicology

In addition to the microbial effects described here, the studied sediment amendments, especially the organics acetate and chitin, affected the survival or growth rates of various macro-organisms under controlled laboratory exposure (Rosen et al., companion submission). Significant lower survival rates and growth rates of tested organisms indicate both mortality and chronic toxicity effects from the amendments such as acetate, chitin, and chitin+apatite. Reduced dissolved oxygen (acetate treatments) or excess ammonia (chitin treatments) produced by increased microbial cell densities or microbial degradation of the amendments aided in explaining the toxicological effects. It was likely the increased microbial cell numbers and/or microbial community shift that deteriorated the overlying water quality, which subsequently affected the survival and growth rates of the macro-organisms. Meanwhile, we could not exclude the possibility that microbial pathogens were enriched by the amendments and directly infected the tested animals. However, this was out of the scope of current work and thus was not included in further discussion.

5. Conclusions

The most probable number technique demonstrated that geochemically important bacterial groups including SRB and MRB were present in the sediments under amendment conditions and could play important roles in metal bioremediation and organic degradation processes. XAS analysis demonstrated that under chitin conditions, there was a modest modification of zinc speciation (increase in $ZnCO_3$), very likely due to the induced bacterial population. DGGE analysis showed that typical organisms responsible for these processes were not always dominant in the sediments. However, the DGGE analysis can be a useful tool to

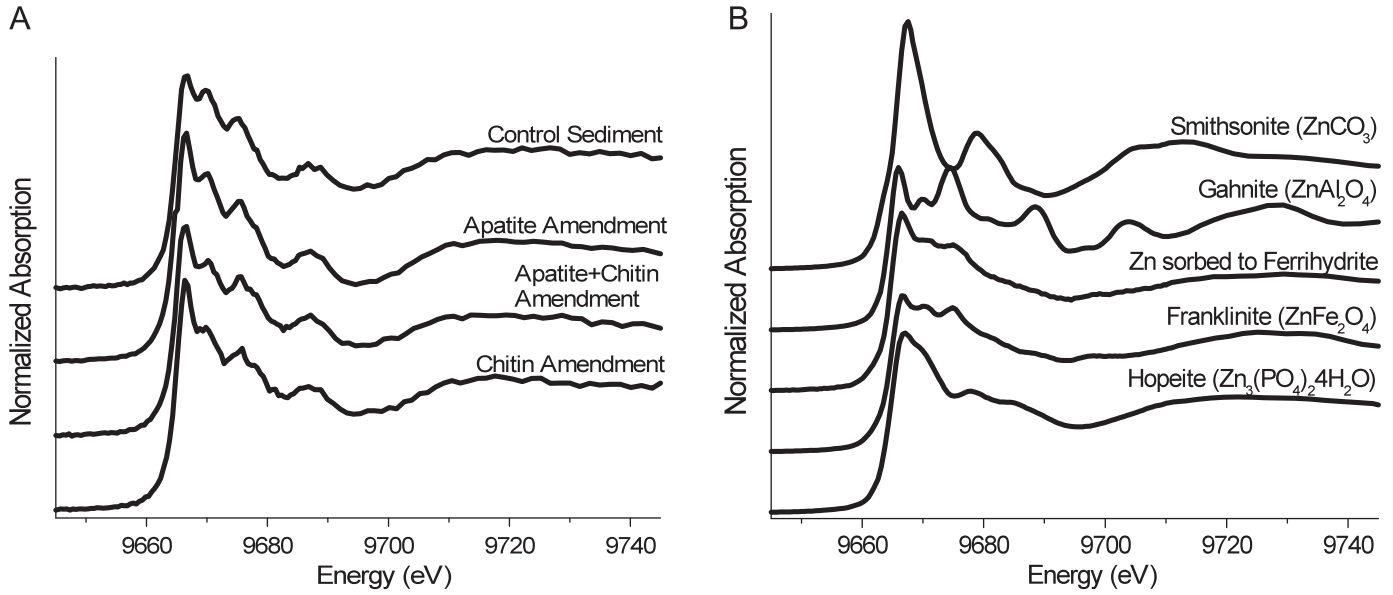

Fig. 6. (A) Normalized K_α Zn X-ray absorption spectra of unamended (control) and amended San Diego Bay sediments. (B) Normalized K_α Zn X-ray absorption spectra of zinc reference materials determined by principal component analysis for linear combination fitting.

Table 3
Linear combination fitting (LCF) results of zinc speciation in unamended and amended San Diego Bay sediments with corresponding edge step intensities and *R*-factors (error).

Sample treatment	Edge step	Zn speciation distribution (%)					*R*-factor[a]
		Smithsonite	Gahnite	Zn sorbed to ferrihydrite	Franklinite	Hopeite	
Control	0.05	9	42	44	5		0.042
Apatite	0.047	14	40	26		21	0.062
Apatite+Chitin	0.048	24	41	27		8	0.065
Chitin	0.051	34	52	14			0.085

[a] *R*-factor=$[(\text{data-fit})^2]/[\text{data}^2]$.

visualize the distinct bacterial populations in overlying water and sediments that were enriched by organic amendments including acetate and chitin. This data was corroborated with the bacterial cell counts and the XAS analysis. DGGE analyses of the sedimentary bacteria did make evident that treatments with organoclay, apatite+organoclay, acetate, and chitin induced significant changes in the bacterial population and reshaped the microbial population structures in both the overlying water and in the sediment horizons. These results emphasize the benefit of performing cultivation-dependent and cultivation-independent concurrent analyses.

Due to the potential water quality and toxicology effects on the macro-benthic communities (see Rosen et al., companion submission), the tendency to be easily dissolved upon exposure to seawater, and cost effectiveness, acetate is not recommended for further investigation as a sediment amendment for contaminated sediments. In contrast, chitin, a naturally occurring and abundant structural polysaccharide in crustaceans, provides a potential for practical application in sediment amendments for organic and inorganic contaminants. However, we recommend further efforts on investigating the environmental safety effects of chitin amendments and optimizing the concentration to apply in marine sediments. Naturally, there will be differences based on processes such as current and flow, shallow or deep, and stagnant waters verses fast-moving water bodies. In conclusion, incorporation of apatite and chitin sediment amendments at select sites may be one of the preferred options of in situ remediation of heavy metal and organic contaminated sediments.

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